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In silico whole-transcriptome analysis reveals a potential hsa_circ_0000375-miR-424-5p-TPM2/SRPX/SRGAP1 regulatory network related to liver metastasis of colorectal cancer

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ABSTRACT

Liver metastasis is the main lethal cause of colorectal cancer (CRC). The knowledge of role and mechanism of circular RNA (circRNA) in liver metastasis of CRC is still inadequate. In this study, whole-transcriptome analysis was performed using three datasets (GSE147597, GSE147602 and GSE147603). A total of 14 potential circRNAs were identified, after which their structural patterns and binding miRNAs were obtained. Next, 45 differentially expressed miRNAs (DEmiRNAs) between CRC without and with liver metastasis were acquired, consisting 38 upregulated and 7 downregulated miRNAs. After conducting intersection analysis, expression validation and correlation analysis, miR-761 and miR-424-5p were selected as the most potential miRNAs linked to liver metastasis of CRC. Subsequently, the target genes of miR-761 or miR-424-5p were predicted and differentially expressed genes (DEGs) between CRC without and with liver metastasis were obtained. 257 genes that were commonly appeared in predicted genes and DEGs were significantly enriched in "epithelial-to-mesenchymal transition" and "signaling by Robo receptor". Among these enriched genes, only TPM2, SRPX and SRGAP1 were significantly negatively correlated with miR-424-5p and were positively linked to hsa_circ_0000375 in CRC without or with liver metastasis. Collectively, the current findings elucidated a potential hsa_circ_0000375-miR-424-5p-TPM2/SRPX/SRGAP1 network contributing to liver metastasis of CRC.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies and ranks the third cause of cancer-related deaths all over the worldwide [1,2]. Liver is the leading target organ of blood metastasis of CRC. As reported, nearly 20 % of patients with CRC present synchronous liver metastasis, and another 20 % CRC patients develop liver metastasis within 5 years after early diagnosis [3]. Furthermore, liver metastasis is the main lethal reason of CRC, with the five-year overall survival less than 15 % [4]. Despite the molecular mechanism of liver metastasis of CRC has been strenuously studied, its knowledge is still inadequate and need to be further explored.

Circular RNAs (circRNAs), a group of novel, endogenous and non-coding RNAs, have covalently closed loops without 5′-cap and 3′-polyadenylated tail structure which makes them more stable than linear counterparts [5–7]. Recently, the critical roles of circRNAs in

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initiation and progression of human malignancies have been gradually unlocked, including CRC [8,9]. For example, Liu et al. confirmed that circIFT80 enhanced the progression of CRC by targeting beta-catenin [10]; Fan et al. showed that circ_0000395 facilitated CRC development *via* upregulating MYH9 [11]. However, the specific role and mechanism of circRNA in liver metastasis of CRC have not been fully elucidated and deserve to be further studied.

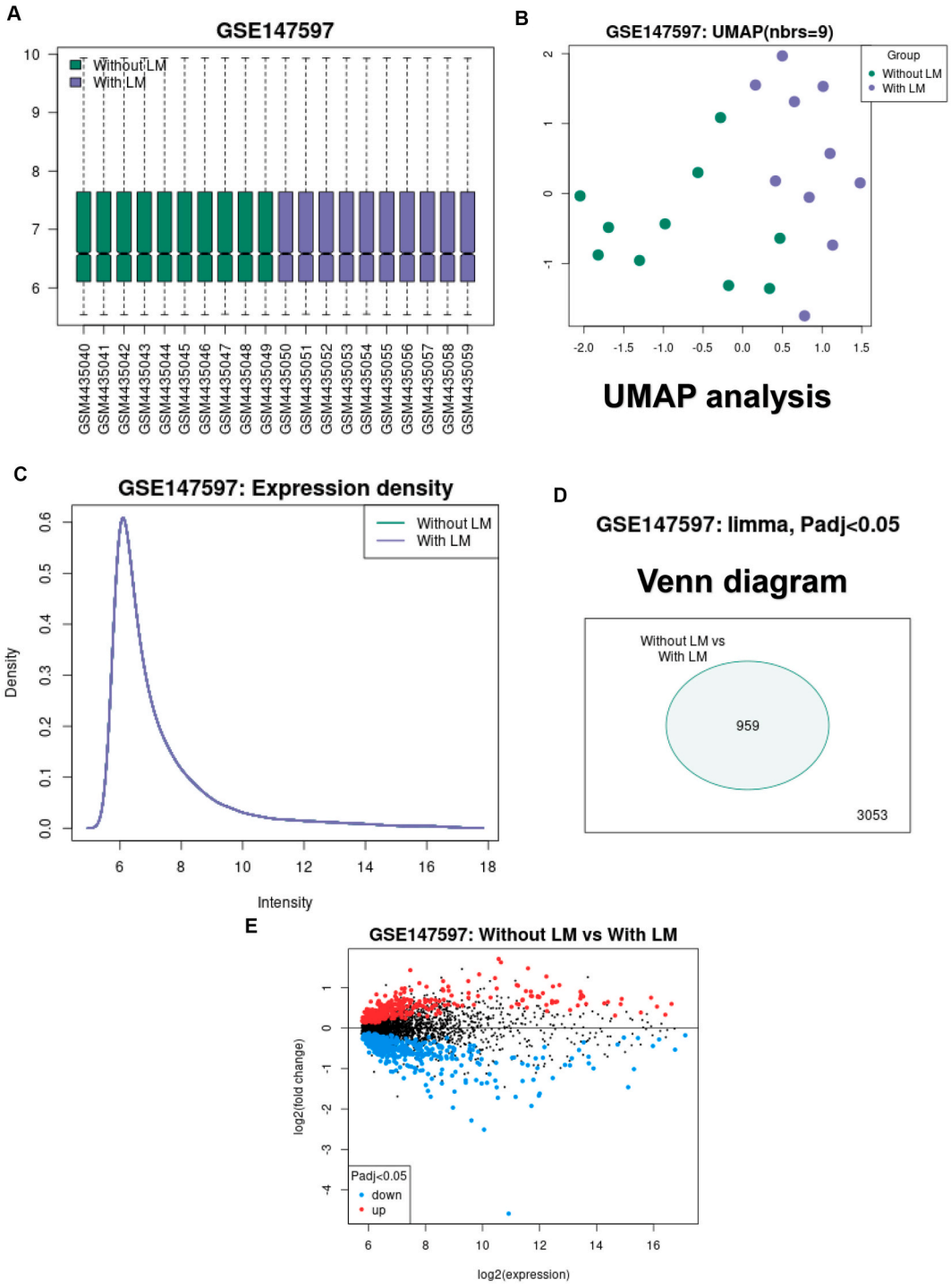


Fig. 1. Screening differentially expressed circRNAs (DECs) between CRC without liver metastasis and with liver metastasis. (A) The normalization of the 20 tissue samples in GSE147597 by GEO2R. (B) The UMAP analytic result of GSE147597 by GEO2R. (C) The expression density of GSE147597. (D) The significant DECs between CRC without and with liver metastasis. (E) The volcano plot of the DECs between CRC without and with liver metastasis in GSE147597.

In our previous work [12], a potential hsa_circ_0001955/hsa_circ_0000977-mediated miRNA-mRNA regulatory network involved in the transition from normal tissues to CRC was established by whole-transcriptome analysis. The oncogenic role of hsa_circ_0001955 in CRC was following validated by Gao et al. [13]. In this study, we aimed to identify the potential circRNA-miRNA-mRNA network contributing to liver metastasis of CRC. The findings from the current study might provide crucial clues for understanding the molecular mechanism of CRC liver metastasis and developing effective therapeutic targets in treating liver metastasis of CRC.

2. Results

2.1. Screen of 14 potential circRNAs associated with liver metastasis of CRC

To study the role and mechanism of circRNA in liver metastasis of CRC, a circRNA dataset GSE147597 was firstly employed. GSE147597 contained 10 CRC patients without liver metastasis and 10 CRC patients with liver metastasis. No statistical difference of baseline of age between the two groups was observed (without liver metastasis: 64.9 ± 1.853; with liver metastasis: 61.9 ± 4.905; $P = 0.574$). Using GEO2R online tool, data normalization, UMAP analysis and expression density were successively performed (Fig. 1A–C). A total of 959 significant differentially expressed circRNAs (DECs), consisting of 432 upregulated and 527 downregulated DECs in CRC without liver metastasis, were identified (Fig. 1D–E). Furthermore, 14 DECs with |Fold change|>1.5 were chosen for subsequent analysis. As shown in Table 1 and Fig. 2A-N, 2 DECs were significantly upregulated whereas 12 DECs were markedly downregulated in CRC without liver metastasis compared with CRC with liver metastasis. The 14 circRNAs were considered as the potential circRNAs associated with liver metastasis of CRC.

2.2. Prediction of binding miRNAs of the circRNAs associated with liver metastasis of CRC

The corresponding genome location and parental genes of 14 potential circRNAs were obtained from circBase database (Table 2). It has been widely acknowledged that circRNAs exert their biological functions by competitively binding to miRNAs, at least partially [14]. The structural patterns of potential circRNAs were drew by CSCD database. Consequently, the structural loops of 11 circRNAs were presented in Fig. 3A-L. Intriguingly, all the 11 circRNAs had microRNA response elements (MREs). Subsequently, the possible miRNAs that could bind to these circRNAs were predicted. For better visualization, a circRNA-miRNA regulatory network was established using Cytoscape software as shown in Fig. 4.

2.3. Identification of potential miRNAs associated with liver metastasis of CRC

To further identify the potential miRNAs linked to liver metastasis of CRC, a miRNA dataset GSE147603 possessed identical CRC patients with GSE147597 was introduced. Differential expression analysis for GSE147603 was performed. As presented in Fig. 5A–B, a total of 45 differentially expressed miRNAs (DEmiRNAs) between CRC without and with liver metastasis were acquired, including 38 upregulated and 7 downregulated DEmiRNAs. Next, intersection analysis for the predicted miRNAs of DECs and DEmiRNAs was conducted. As suggested in Fig. 5C, none of miRNAs that were commonly appeared in “Predicted miRNA” and “DEmiRNA” sets were found. Intriguingly, a total of 7 miRNAs were commonly appeared in both the two miRNA sets (Fig. 5D). As presented in Fig. 6A–G, all the 7 miRNAs were significantly downregulated in CRC with liver metastasis when compared with CRC without liver metastasis, which was identical with the differential expression analysis by GEO2R online tool. Correlation analysis for circRNA-miRNA pairs was performed (Fig. 7A-L). The results revealed that miR-761 was significantly negatively correlated with hsa_circ_0043278 (Fig. 7A), hsa_circ_0006220 (Fig. 7B), hsa_circ_0088045 (Fig. 7C), hsa_circ_0088046 (Fig. 7D), hsa_circ_0000375 (Fig. 7E), as well as miR-424-5p was markedly inversely associated with hsa_circ_0000375 (Fig. 7G) in CRC without or with liver metastasis. Taken together, these findings suggested that miR-761 and miR-424-5p might act as key tumor suppressors in negatively mediating liver metastasis of CRC.

Table 1
The potential differentially expressed circRNAs (DECs) between CRC without liver metastasis and CRC with liver metastasis.

circBase ID	Adjusted P-value	t	B	Log_2FC[a]
hsa_circ_0003270	0.006	4.469	0.599	1.709
hsa_circ_0082182	0.004	4.814	1.366	1.626
hsa_circ_0043278	0.003	−5.092	1.983	−4.587
hsa_circ_0006220	0.030	−3.123	−2.374	−2.510
hsa_circ_0001666	0.029	−3.156	−2.304	−2.284
hsa_circ_0006502	0.010	−3.963	−0.533	−1.968
hsa_circ_0088045	0.013	−3.770	−0.963	−1.925
hsa_circ_0092330	0.002	−5.540	2.960	−1.725
hsa_circ_0088046	0.014	−3.721	−1.073	−1.703
hsa_circ_0000375	0.008	−4.269	0.151	−1.698
hsa_circ_0080210	0.011	−3.854	−0.777	−1.671
hsa_circ_0092289	0.004	−4.918	1.598	−1.620
hsa_circ_0064644	0.016	−3.601	−1.337	−1.572
hsa_circ_0042435	0.003	−5.221	2.266	−1.556

[a] FC: CRC without liver metastasis vs. CRC with liver metastasis (without/with).

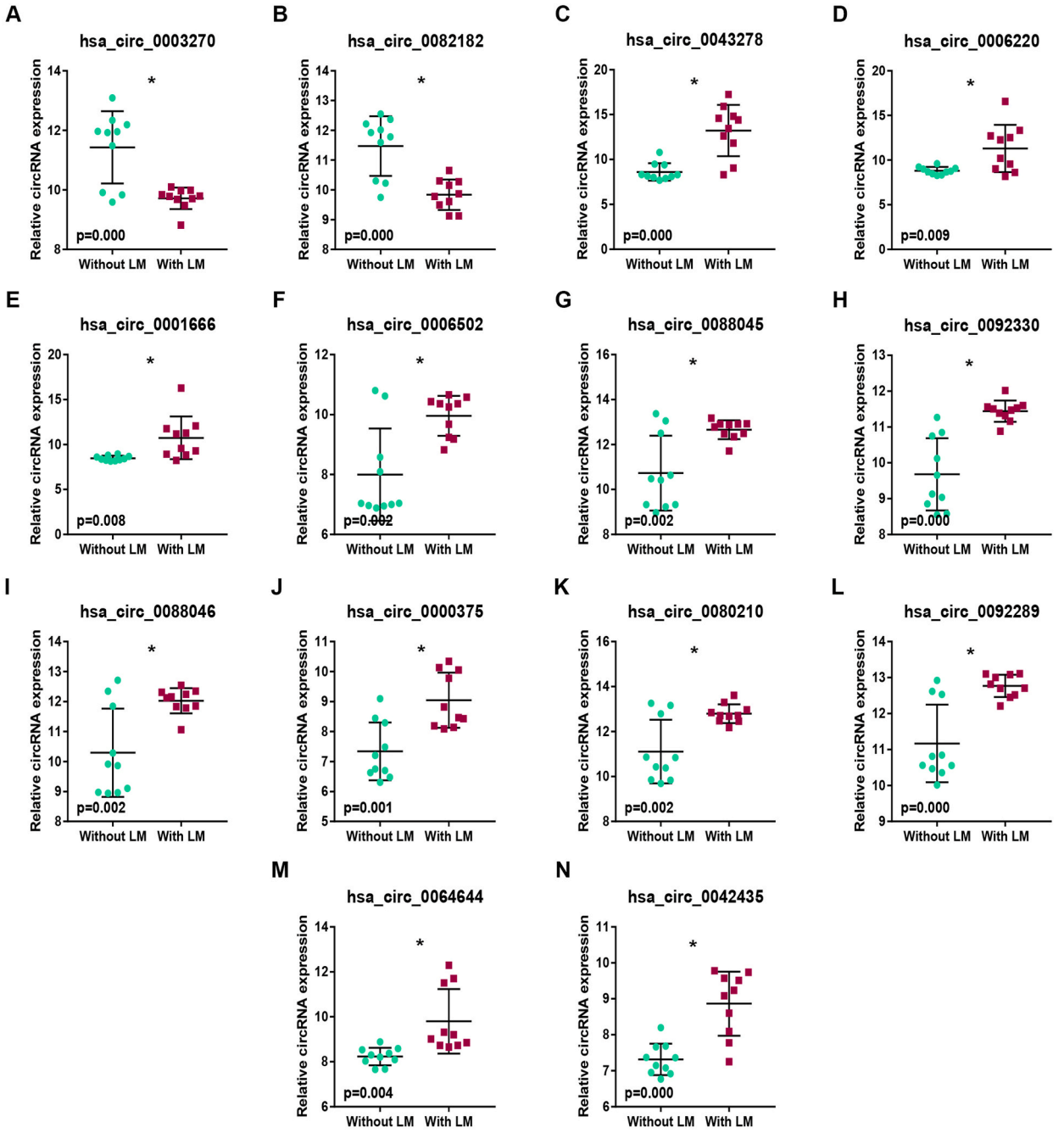


Fig. 2. Expression determination of candidate circRNAs associated with liver metastasis of CRC. The expression levels of hsa_circ_0003270 (A), hsa_circ_0082182 (B), hsa_circ_0043278 (C), hsa_circ_0006220 (D), hsa_circ_0001666 (E), hsa_circ_0006502 (F), hsa_circ_0088045 (G), hsa_circ_0092330 (H), hsa_circ_0088046 (I), hsa_circ_0000375 (J), hsa_circ_0080210 (K), hsa_circ_0092289 (L), hsa_circ_0064644 (M) and hsa_circ_0042435 (N) in CRC without liver metastasis compared with CRC with liver metastasis. *$P < 0.05$.

2.4. Prediction and enrichment analysis of target genes of miRNAs associated with liver metastasis of CRC

Next, the downstream genes of the potential circRNA/miRNA network were explored. GSE147602 was used to obtain differentially expressed genes (DEGs) between CRC without and with liver metastasis. As shown in Fig. 8A–B, the data normalization and differential expression analysis were performed. Next, the target genes of miR-761 or miR-424-5p were predicted. As presented in Figs. 8C, 159 and 1731 target genes were predicted to potentially bind to miR-761 and miR-424-5p, respectively. Furthermore, intersection analysis was performed to acquire the genes that were commonly appeared in "Predicted gene" and "DEG" gene sets, and 257 common genes

Table 2
The locations and parental genes of candidate circRNAs.

circBase ID	Location	Parental gene
hsa_circ_0003270	chr9:128,099,296-128,099,870	GAPVD1
hsa_circ_0082182	chr7:128,317,617-128,323,309	FAM71F2
hsa_circ_0043278	chr17:35,797,838–35,800,763	TADA2A
hsa_circ_0006220	chr17:35,800,605–35,800,763	TADA2A
hsa_circ_0001666	chr6:170,626,457-170,639,638	FAM120B
hsa_circ_0006502	chr9:140,458,885-140,459,606	WDR85
hsa_circ_0088045	chr9:114,860,749-114,864,565	SUSD1
hsa_circ_0092330	chr22:19,965,129–19,965,469	ARVCF
hsa_circ_0088046	chr9:114,860,749-114,875,148	SUSD1
hsa_circ_0000375	chr12:6,657,590–6657991	IFFO1
hsa_circ_0080210	chr7:50,737,418-50773020	GRB10
hsa_circ_0092289	chr20:17,595,565–17,595,865	RRBP1
hsa_circ_0064644	chr3:29,910,348-29,941,246	RBMS3
hsa_circ_0042435	chr17:20,149,238–20209395	SPECC1

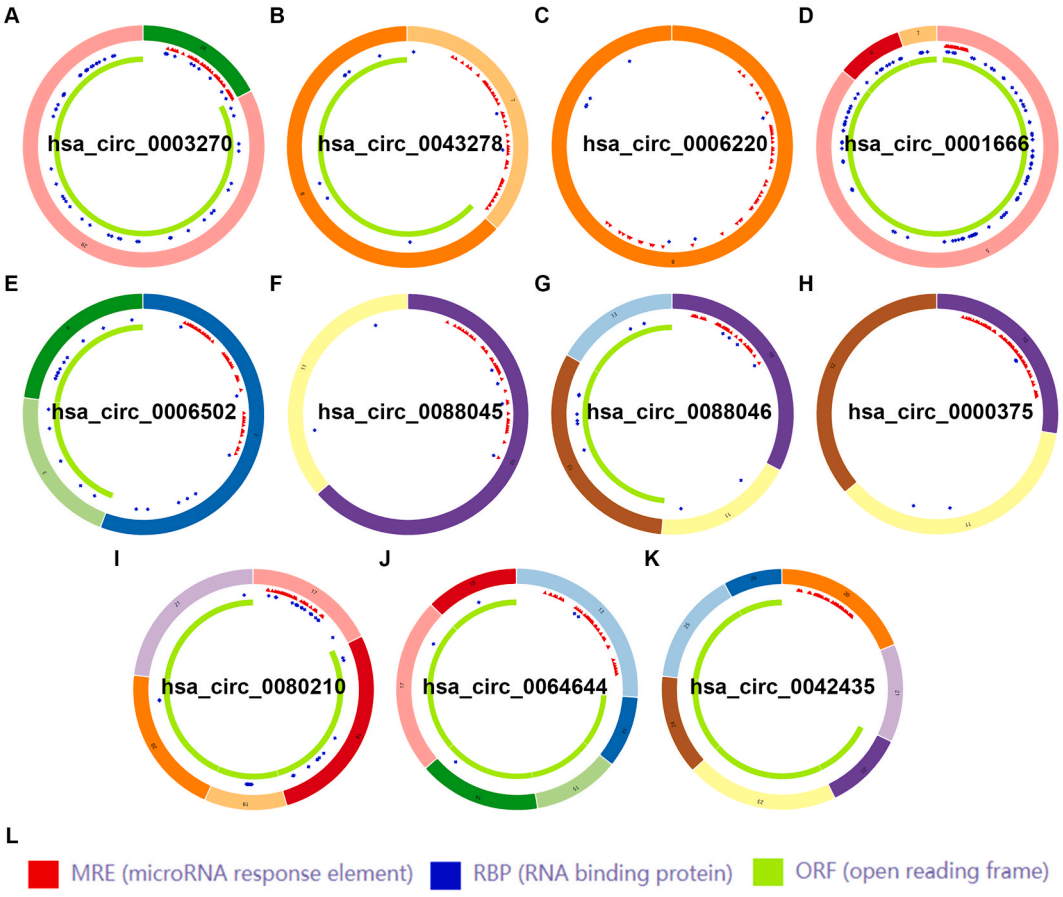


Fig. 3. The structural patterns of candidate circRNAs acquired from CSCD database. The structural pattern of hsa_circ_0003270 (A), hsa_circ_0043278 (B), hsa_circ_0006220 (C), hsa_circ_0001666 (D), hsa_circ_0006502 (E), hsa_circ_0088045 (F), hsa_circ_0088046 (G), hsa_circ_0000375 (H), hsa_circ_0080210 (I), hsa_circ_0064644 (J) and hsa_circ_0042435 (K). (L) The representation of MRE, RBP and ORF.

were identified (Fig. 8D). As shown in Fig. 8E, these common genes were significantly enriched in two biological processes, consisting of "epithelial-to-mesenchymal transition" and "signaling by Robo receptor". 12 (AKT3, SLC2A3, FERMT2, CYP1B1, STON1, TRPC1, SLIT2, QKI, TPM2, RECK, AP1S2 and SRPX) and 5 (ABL2, ENAH, SRGAP1, SLIT2 and CAP2) genes were presented in "epithelial-to-mesenchymal transition" and "signaling by Robo receptor", respectively. As presented in Fig. 9A-P, all the genes were markedly upregulated in CRC with liver metastasis when compared with CRC without liver metastasis, which was identical with the result from differential expression analysis. These findings indicated that these enriched genes might function as oncogenes involved in the process

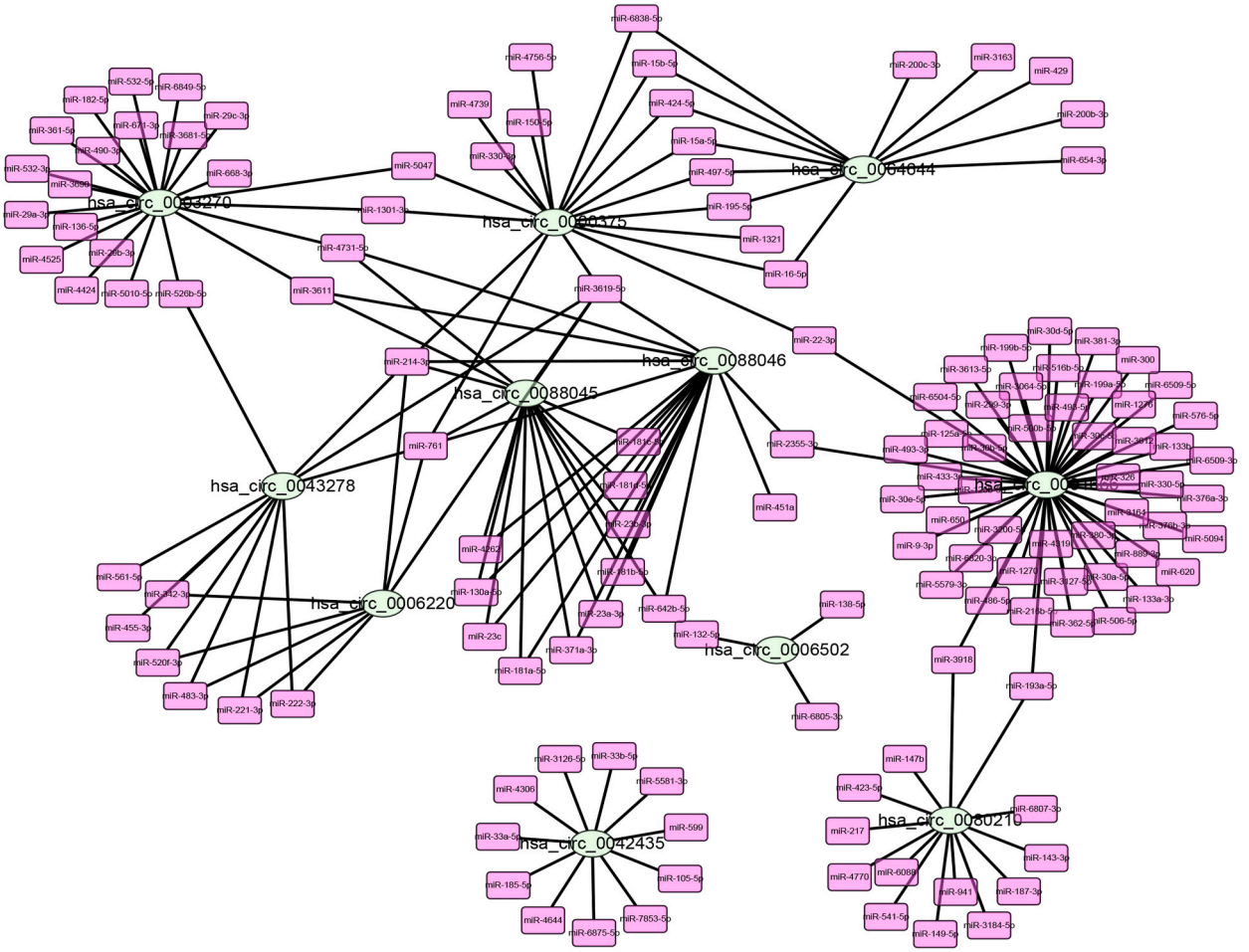


Fig. 4. Establishment of a potential circRNA-miRNA regulatory network by Cytoscape software.

of liver metastasis of CRC.

2.5. Construction of a potential circRNA-miRNA-mRNA network contributing to liver metastasis of CRC

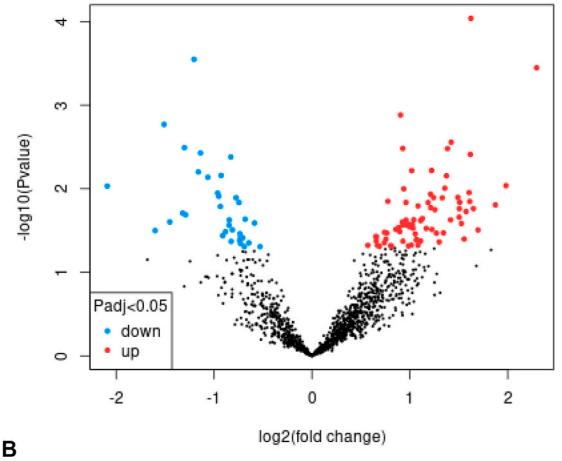
By matching the miRNA-gene prediction pairs, we found that all the potential genes appeared in "epithelial-to-mesenchymal transition" or "signaling by Robo receptor" were only potentially bound to miR-424-5p rather than miR-761. According to action mechanism of miRNA, there should be inverse expression correlation between miRNAs and their target genes. As shown in Fig. 10A-P, miR-424-5p was significantly inversely correlated with AKT3, SLC2A3, FERMT2, CYP1B1, STON1, TRPC1, SLIT2, QKI, TPM2, RECK, SRPX, ABL2 or SRGAP1 in CRC without or with liver metastasis. Moreover, based on competing endogenous RNA (ceRNA) hypothesis, there should be positive expression relationship between circRNAs and their corresponding binding genes. As presented in Fig. 11A-P, only three circRNA-gene pairs, consisting of hsa_circ_0000375/TPM2 (Fig. 11I), hsa_circ_0000375/SRPX (Fig. 11L) and hsa_circ_0000375/SRGAP1 (Fig. 11O), presented positive expression correlation with statistical significance. Knockdown of hsa_circ_0000375 could decrease the migrated ability of CRC cells (Fig. 12A–B). The inhibitory effect could be reversed by suppression of miR-424-5p in CRC cells (Fig. 12C). Furthermore, inhibition of hsa_circ_0000375 restrained expression of TPM2, SRPX or SRGAP1, which could be weakened by knockdown of miR-424-5p in CRC cells (Fig. 12D–F). All these findings demonstrated that hsa_0000375-miR-424-5p-TPM2/SRPX/SRGAP1 might be a potential regulatory network contributing to liver metastasis of CRC (Fig. 12G).

3. Discussion

Liver metastasis, the primary lethal cause of CRC, accounts for its poor prognosis. During the past decades, increasing lines of evidence have reported the key roles of circRNAs in cancer initiation and progression, including CRC. However, its knowledge of circRNAs in liver metastasis of CRC is still insufficient and need to be further elucidated.

In this study, a series of datasets regarding liver metastasis of CRC from 20 identical CRC patients without or with liver metastasis

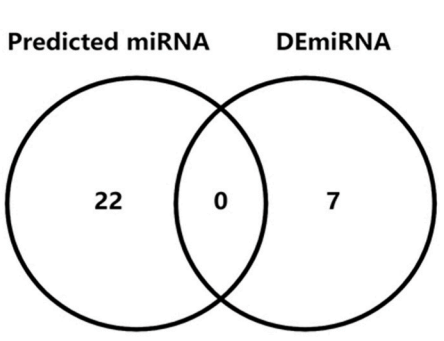
A



B

miRNA name	Adjusted P-value	t	B	logFC
miR-33b-5p	0.000	4.185	-0.181	2.295
miR-199b-5p	0.031	2.296	-3.462	1.696
miR-142-5p	0.000	4.731	0.793	1.623
miR-32-5p	0.011	2.761	-2.709	1.605
miR-301a-5p	0.019	2.529	-3.093	1.573
miR-20a-3p	0.040	2.178	-3.642	1.554
miR-3152-5p	0.022	2.458	-3.208	1.503
miR-144-3p	0.013	2.703	-2.807	1.490
miR-519e-3p	0.003	3.351	-1.683	1.422
miR-33a-5p	0.003	3.277	-1.814	1.384
miR-2681-3p	0.007	2.961	-2.369	1.374
let-7i-3p	0.034	2.260	-3.518	1.343
miR-429	0.043	2.139	-3.700	1.300
miR-223-3p	0.034	2.256	-3.524	1.270
miR-147b	0.018	2.552	-3.056	1.255
miR-191-5p	0.006	3.024	-2.260	1.221
miR-345-5p	0.012	2.742	-2.741	1.211
miR-3607-5p	0.030	2.316	-3.432	1.167
miR-127-3p	0.016	2.598	-2.981	1.084
miR-761	0.041	2.165	-3.661	1.066
miR-3649	0.035	2.245	-3.541	1.055
miR-1284	0.030	2.321	-3.424	1.031
miR-424-5p	0.027	2.364	-3.356	1.025
miR-3676-5p	0.006	3.022	-2.262	1.018
miR-148a-3p	0.028	2.340	-3.395	0.994
miR-122-3p	0.048	2.085	-3.779	0.985
miR-1307-5p	0.024	2.422	-3.265	0.960
miR-2115-3p	0.010	2.807	-2.632	0.937
miR-155-5p	0.027	2.363	-3.359	0.928
miR-34a-5p	0.025	2.398	-3.303	0.923
miR-302e	0.001	3.658	-1.133	0.905
miR-425-5p	0.033	2.274	-3.496	0.897
miR-937	0.029	2.327	-3.415	0.886
miR-548an	0.014	2.655	-2.886	0.775
miR-193a-3p	0.040	2.177	-3.644	0.754
miR-148b-3p	0.033	2.266	-3.509	0.740
miR-3195	0.048	2.093	-3.768	0.669
miR-21-3p	0.048	2.094	-3.767	0.570
miR-1587	0.039	-2.193	-3.620	-0.707
miR-1260b	0.004	-3.181	-1.984	-0.829
miR-3201	0.027	-2.358	-3.367	-0.847
miR-4644	0.004	-3.228	-1.902	-1.139
miR-4708-3p	0.000	-4.279	-0.013	-1.206
miR-125b-1-3p	0.025	-2.399	-3.301	-1.453
miR-4516	0.002	-3.553	-1.322	-1.512

C



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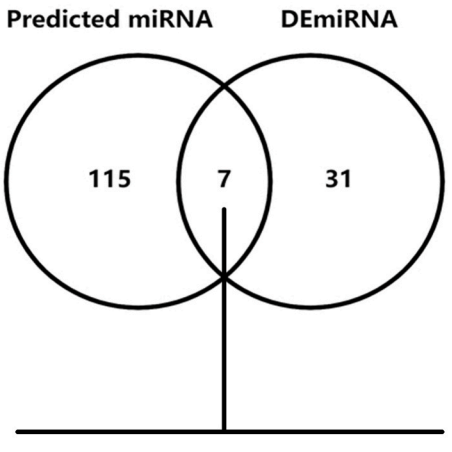

miR-761

miR-199b-5p

miR-424-5p

miR-147b

miR-429

miR-33a-5p

miR-33b-5p

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Fig. 5. Identification of potential miRNAs related to liver metastasis of CRC. (A) The volcano plot of the differentially expressed miRNAs (DEmiRNAs) between CRC without and with liver metastasis in GSE147603. (B) The expressed landscape of significant DEmiRNAs between CRC without and with liver metastasis in GSE147603. "Red" and "Green" indicated that miRNAs were significantly upregulated and downregulated in CRC without and with liver metastasis compared with CRC with and with liver metastasis, respectively. (C) The intersection of predicted miRNAs of circRNAs (upregulated in CRC without liver metastasis) and DEmiRNAs (downregulated in CRC without liver metastasis). (D) The intersection of predicted miRNAs of circRNAs (downregulated in CRC without liver metastasis) and DEmiRNAs (upregulated in CRC without liver metastasis). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

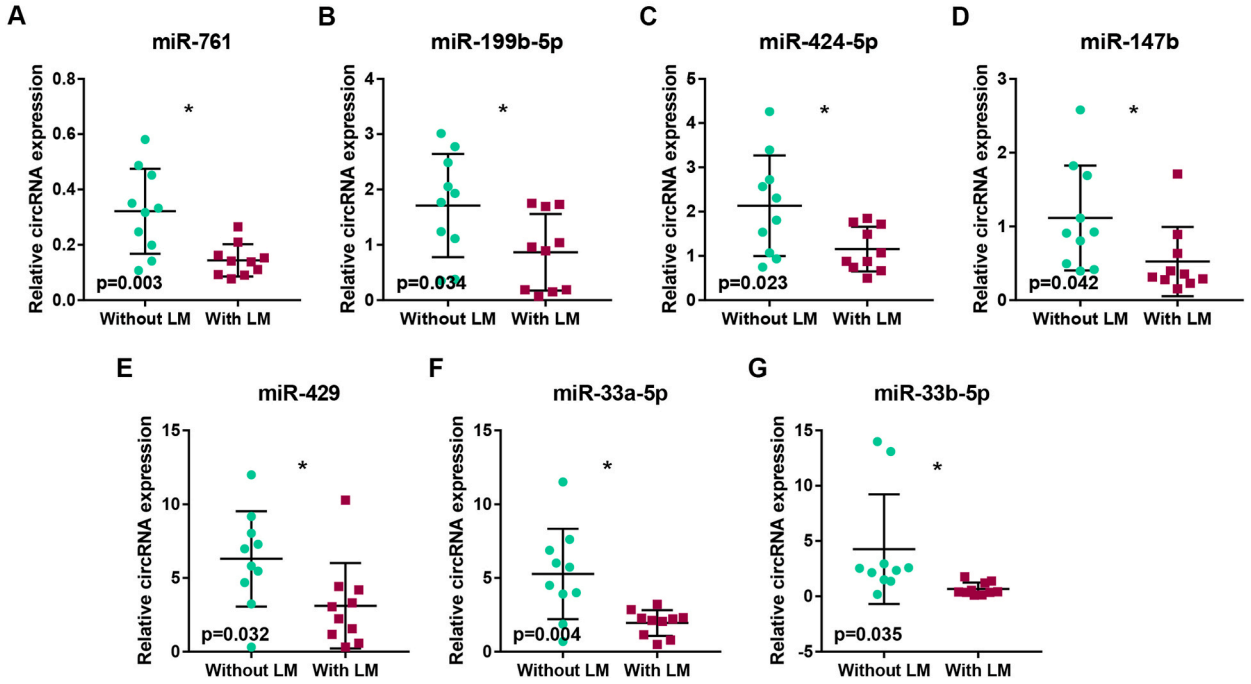

Fig. 6. Expression determination of candidate miRNAs related to liver metastasis of CRC. The expression levels of miR-761 (A), miR-199b-5p (B), miR-424-5p (C), miR-147b (D), miR-429 (E), miR-33a-5p (F) and miR-33b-5p (G) in CRC without liver metastasis compared with CRC with liver metastasis. $*P < 0.05$.

were employed, including circRNA dataset GSE147602, miRNA dataset GSE147603 and mRNA dataset GSE147603.

Firstly, the potential circRNAs that might be involved in liver metastasis of CRC were identified by performing differential expression analysis. Consequently, a total of 14 potential circRNAs were identified. Some of them have been reported to be closely linked to CRC development, diagnosis and prognosis. For example, Dao et al. suggested that hsa_circ_0082182 possessed diagnostic value for CRC [15]; Zhou et al. confirmed that hsa_circ_0001666 inhibited CRC progression by regulating miR-576-5p/PCDH10 axis [16]; Yin et al. suggested that hsa_circ_0000375 was significantly associated with prognosis of patients with CRC [17]. However, none of them have been reported to participate in liver metastasis of CRC.

It has been acknowledged that miRNA sponging mechanism is widely involved in circRNA-mediated diverse biological processes and functions [18–20]. Thus, the potential binding miRNAs of these potential cicRNAs were predicted. Subsequently, the significant DEmiRNAs between CRC without liver metastasis and CRC with liver metastasis were identified, after which intersection analysis was performed. Consequently, a total of seven miRNAs that were commonly appeared in predicted miRNAs of circRNAs and DEmiRNAs, consisting of miR-761, miR-199b-5p, miR-424-5p, miR-147b, miR-429 and miR-33a/b-5p. Previous studies have validated the suppressive roles of some of these miRNAs in liver metastasis of CRC. For example, Zhi et al. showed that miR-761 was involved in circ102049-mediated positive regulation of CRC liver metastasis [21]; Shen et al. showed that downregulation of miR-199b-5p was associated with distant metastasis of CRC [22]; Sun et al. demonstrated that miR-429 suppressed invasion of CRC [23].

Based on ceRNA mechanism [24,25], there should be negative expression correlation between circRNAs and corresponding miRNAs. By performing correlation analysis, only miR-761 and miR-424-5p were significantly inversely correlated with circRNAs (5 for miR-761 and 1 for miR-424-5p) in CRC without or with liver metastasis. Our team and other groups have showed that miRNAs play key roles by negatively regulating downstream target genes [26,27].

For studying the downstream molecular mechanism of the established circRNA/miRNA network associated with liver metastasis of CRC, the target genes of miR-761 or miR-424-5p were predicted. Moreover, the DEGs between CRC without and with liver metastasis were identified using GSE147602 by GEO2R tool. Intersection analysis revealed that 257 genes associated with liver metastasis of CRC were commonly appeared in predicted genes of miRNAs and DEGs. These potential genes were significantly enriched in "epithelial-to-

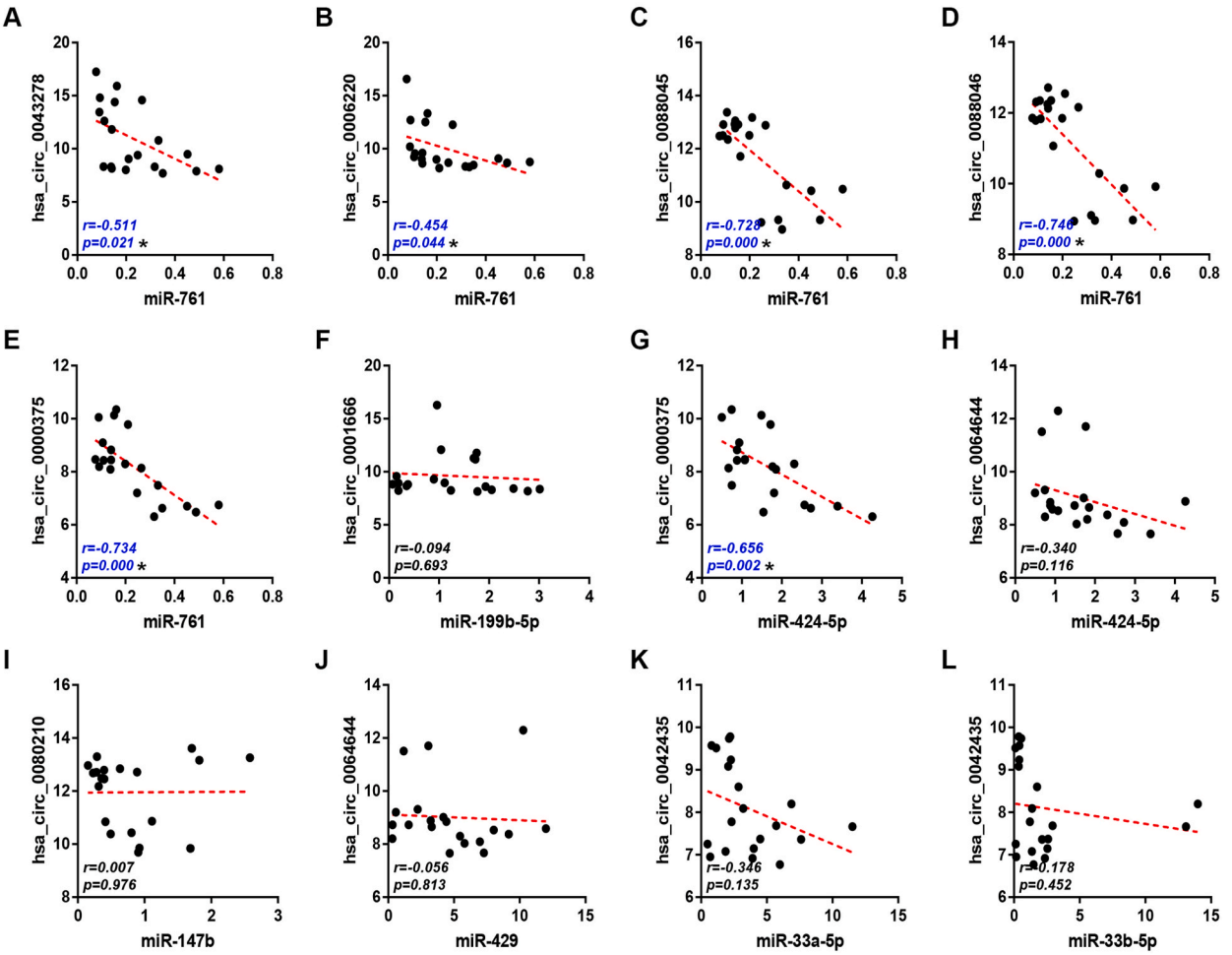

Fig. 7. Correlation analysis for liver metastasis-related circRNA-miRNA pairs in CRC. The expression correlation of hsa_circ_0043278-miR-761 (A), hsa_circ_0006220-miR-761 (B), hsa_circ_0088045-miR-761 (C), hsa_circ_0088046-miR-761 (D), hsa_circ_0000375-miR-761 (E), hsa_circ_0001666-miR-199b-5p (F), hsa_circ_0000375-miR-424-5p (G), hsa_circ_0064644-miR-424-5p (H), hsa_circ_0080210-miR-147b (I), hsa_circ_0064644-miR-429 (J), hsa_circ_0042435-miR-33a-5p (K) and hsa_circ_0043435-miR-33b-5p (L) pairs in CRC without or with liver metastasis.

mesenchymal transition" and "signaling by Robo receptor". Both the two pathways have been widely acknowledged to be closely linked to cancer metastasis [28–30]. The expression levels of these enriched genes were further validated.

Finally, according to miRNA action mechanism and ceRNA hypothesis as mentioned above, correlation analysis for identified miRNA-gene or circRNA-gene pairs was performed in CRC without or with liver metastasis, and three most potential genes were identified, consisting of TPM2, SRPX and SRGAP1 which were significantly positively associated with hsa_circ_0000375 and negatively correlated with miR-424-5p in CRC without or with liver metastasis. Intriguingly, all the three potential genes have been reported to be closely associated with CRC. For example, Yang et al. showed that SRPX possessed cancer-promoting effect in rectal cancer [31]; TPM2 was identified to link to poor prognosis of patients with CRC [32]; SRGAP1 has been also reported to be associated with tumor progression, migration and poor prognosis of CRC [33].

Collectively, the current findings elucidated a potential hsa_circ_0000375-miR-424-5p-TPM2/SRPX/SRGAP1 regulatory network that might contribute to liver metastasis of CRC. Some experiments were conducted to preliminarily validate these findings. The results showed that hsa_circ_0000375 decreased the migrated ability of CRC cells and downregulated the expression levels of TPM2, SRPX and SRGAP1, which could be partially reversed by inhibition of miR-424-5p in CRC cells. However, there were still some limitations in this study: (1) only DECs with |FC| more than 1.5 were included for analysis; (2) the results from this study were based on bioinformatic analysis and *in vitro* cell assays. Therefore, these findings of interest should be further confirmed by much more animal experimental validation and large clinical trials in the future.

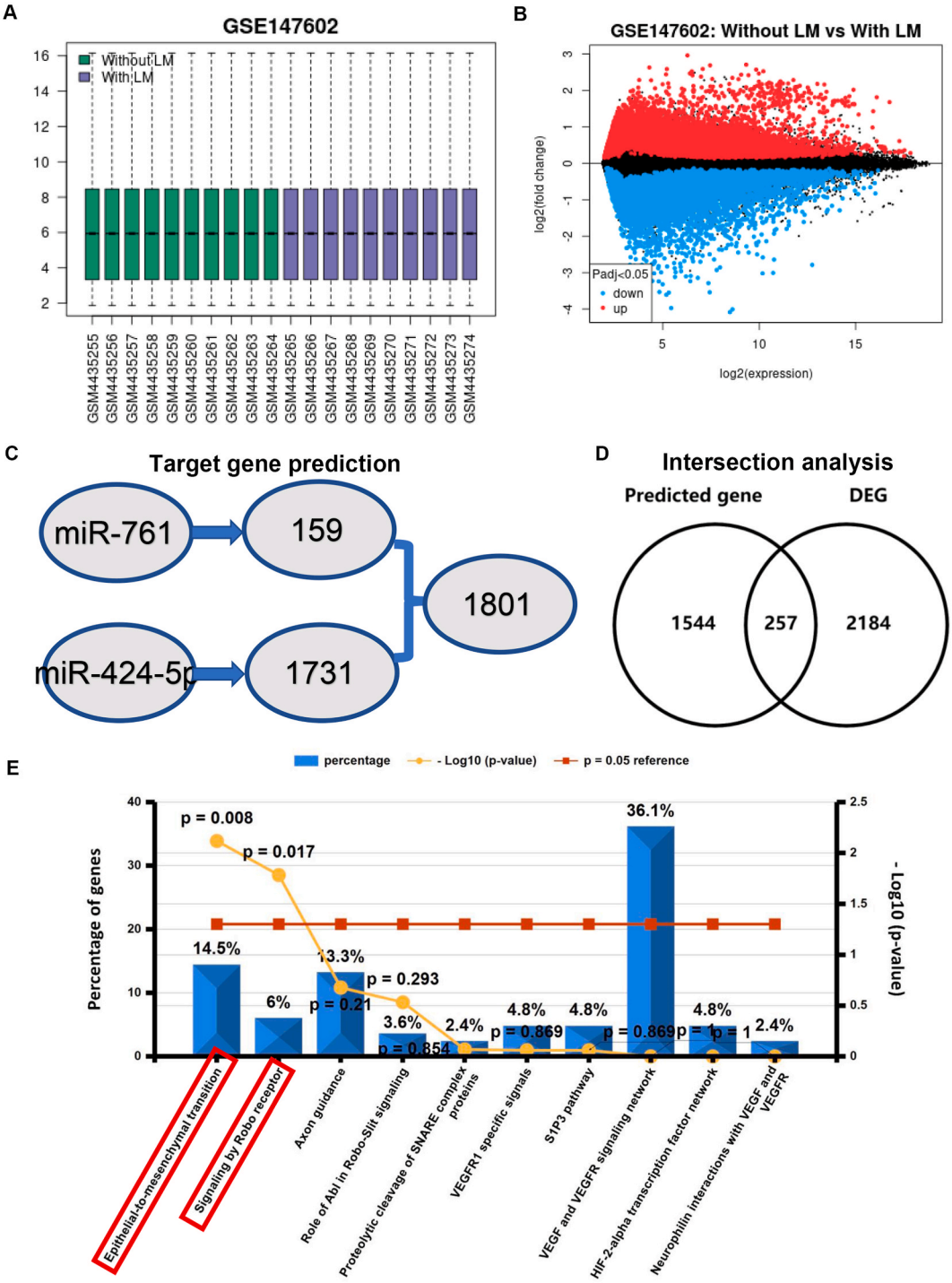

Fig. 8. Identification and analysis for differentially expressed genes (DEGs) associated with liver metastasis of CRC. (A) The normalization of the 20 tissue samples in GSE147602 by GEO2R. (B) The volcano plot of the differentially expressed genes (DEGs) between CRC without and with liver metastasis in GSE147602. (C) The predicted genes of miR-761 and miR-424-5p. (D) The intersection of predicted target genes of miRNAs (miR-761 and miR-424-5p) and significant DEGs (CRC without liver metastasis vs. CRC with liver metastasis). (E) The top 10 enriched biological pathways of the common genes by FunRich software.

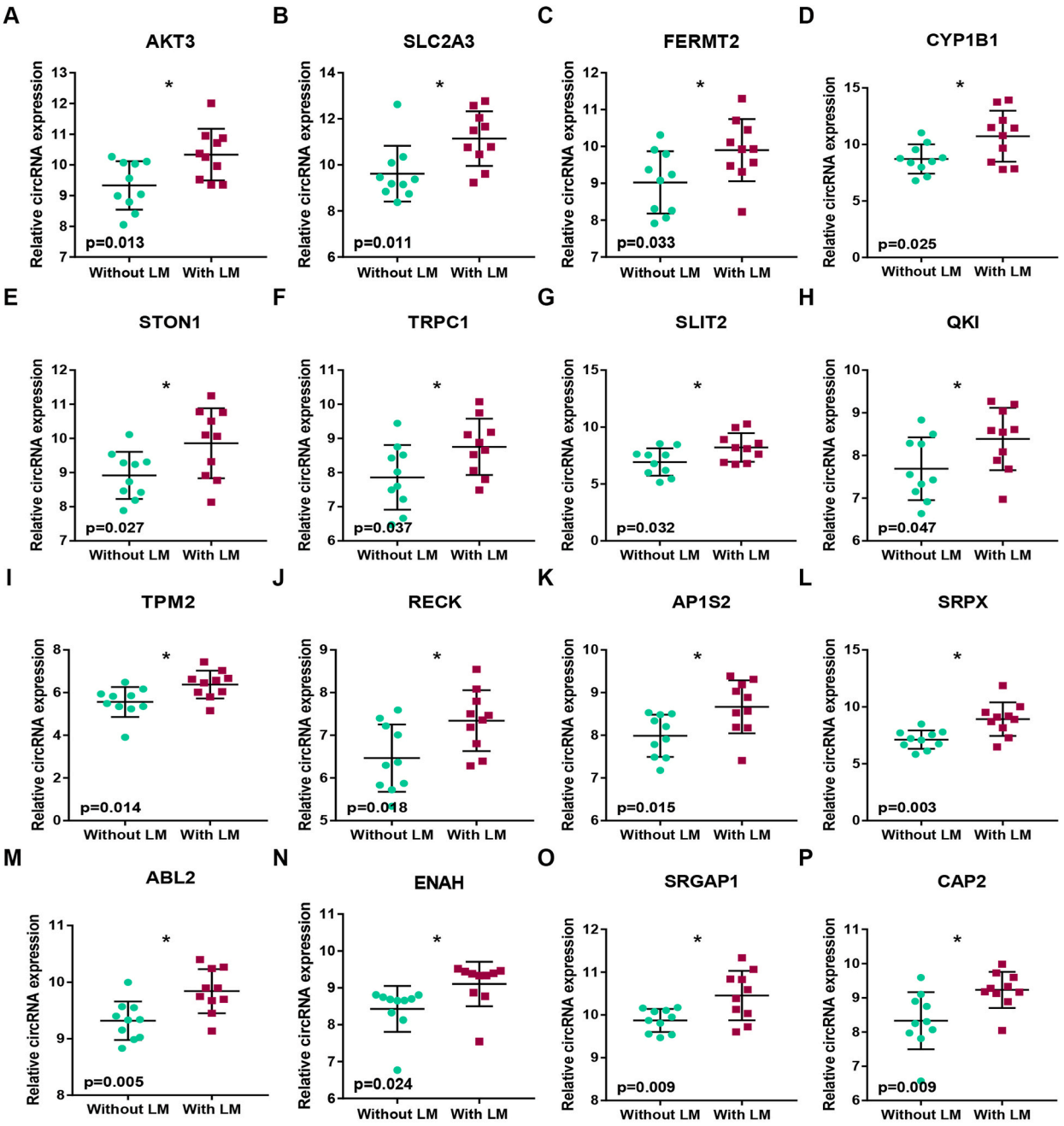

Fig. 9. Expression determination of candidate liver metastasis-related genes in CRC. The expression levels of AKT3 (A), SLC2A3 (B), FERMT2 (C), CYP1B1 (D), STON1 (E), TRPC1 (F), SLIT2 (G), QKI (H), TPM2 (I), RECK (J), AP1S2 (K), SRPX (L), ABL2 (M), ENAH (N), SRGAP1 (O) and CAP2 (P) in CRC without liver metastasis compared with CRC with liver metastasis. *$P < 0.05$.

4. Materials and methods

4.1. Inclusion of datasets

In this study, we aimed to explore the circRNA molecular mechanism involved in liver metastasis of CRC. The NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) was employed to include possible datasets regarding liver metastasis of CRC. The included datasets should meet several selection criteria: (1) studying human body rather than other animals; (2) investigating tissue samples rather other cell lines; (3) containing circRNA, miRNA and mRNA expression profiles. Finally, only one dataset GSE147711 met all these selection criteria, containing three sub-datasets GSE147597 (circRNA profile), GSE147602 (miRNA profile) and GSE147603

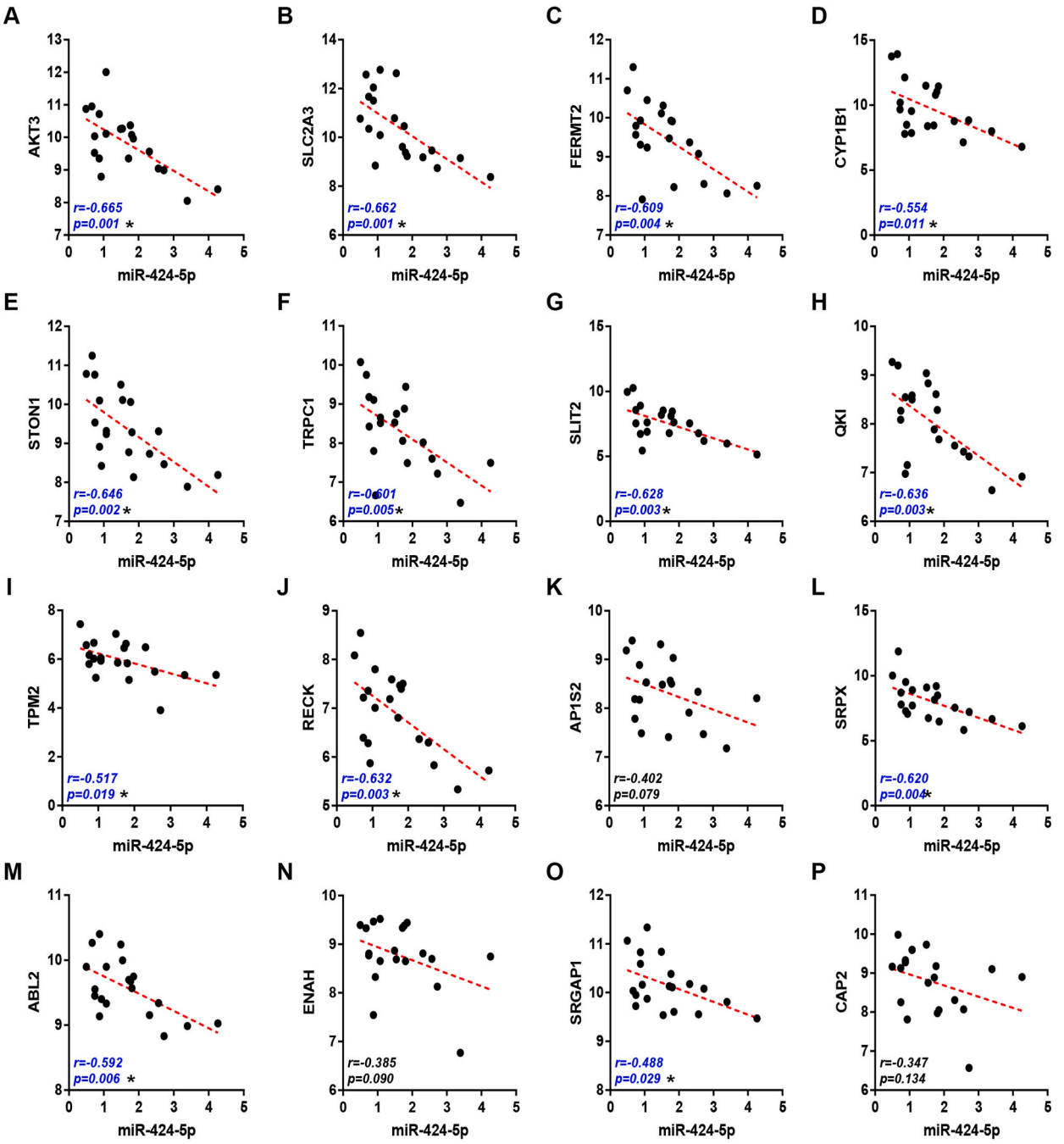

Fig. 10. Correlation analysis for liver metastasis-related miRNA-gene pairs in CRC. The expression correlation of miR-424-5p-AKT3 (A), miR-424-5p-SLC2A3 (B), miR-424-5p-FERMT2 (C), miR-424-5p-CYP1B1 (D), miR-424-5p-STON1 (E), miR-424-5p-TRPC1 (F), miR-424-5p-SLIT2 (G), miR-424-5p-QKI (H), miR-424-5p-TPM2 (I), miR-424-5p-RECK (J), miR-424-5p-AP1S2 (K), miR-424-5p-SRPX (L), miR-424-5p-ABL2 (M), miR-424-5p-ENAH (N), miR-424-5p-SRGAP1 (O) and miR-424-5p-CAP2 (P) pairs in CRC without or with liver metastasis.

(mRNA profile). These datasets had 10 CRC patients without liver metastasis and 10 CRC patients with liver metastasis. All of the three sub-datasets were based on platform of GPL19978 Agilent-069978 Arraystar Human CircRNA microarray V1, GPL21047 Agilent-074348 Human LncRNA v6 4 × 180K and GPL18058 Exiqon miRCURY LNA microRNA array 7th generation, respectively.

4.2Differential expression analysis

The differentially expressed circRNAs (DECs), miRNAs (DEmiRNAs) or genes (DEGs) between CRC without liver metastasis and

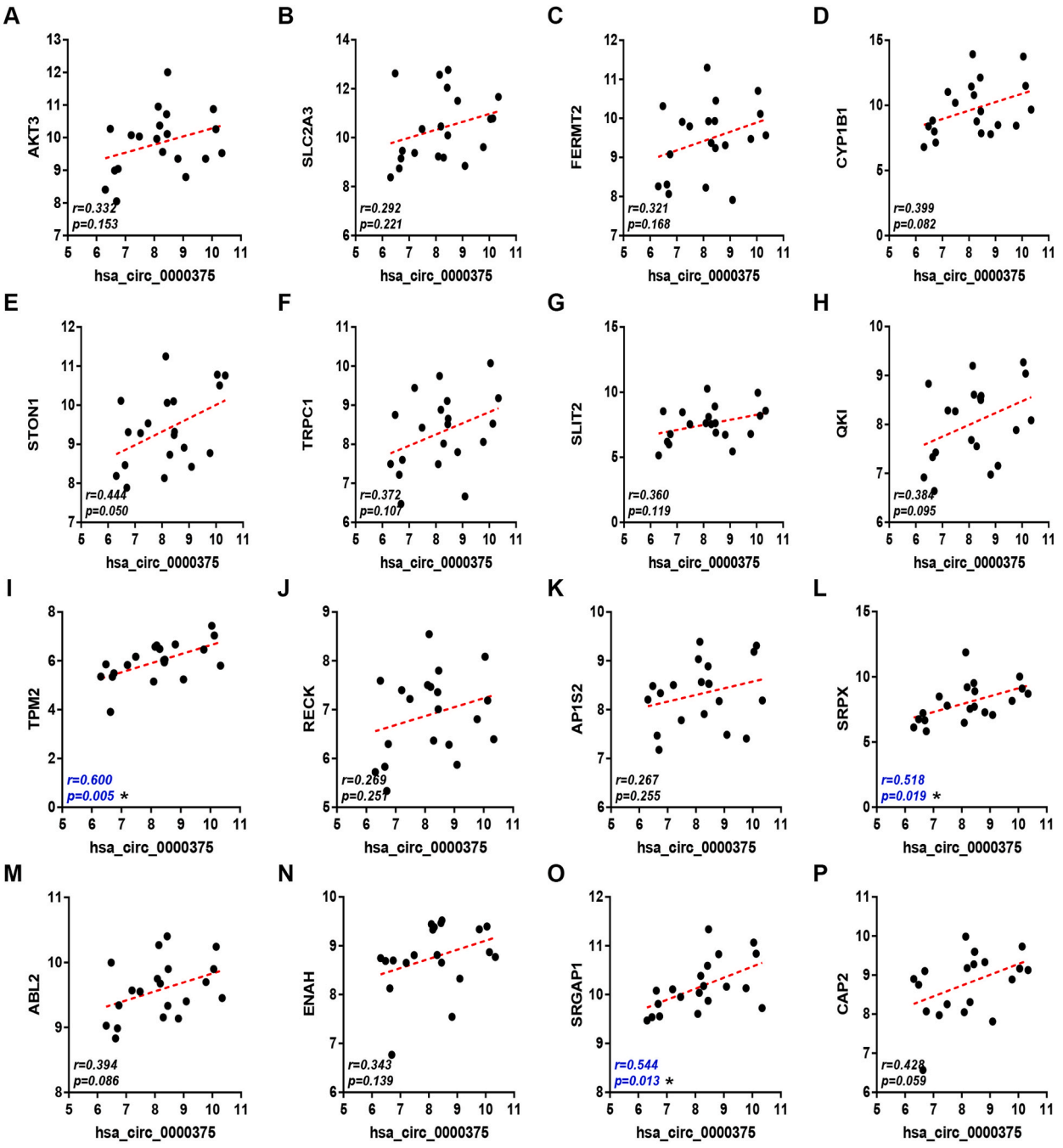

Fig. 11. Correlation analysis for liver metastasis-related circRNA-gene pairs in CRC. The expression correlation of hsa_circ_0000375-AKT3 (A), hsa_circ_0000375-SLC2A3 (B), hsa_circ_0000375-FERMT2 (C), hsa_circ_0000375-CYP1B1 (D), hsa_circ_0000375-STON1 (E), hsa_circ_0000375-TRPC1 (F), hsa_circ_0000375-SLIT2 (G), hsa_circ_0000375-QKI (H), hsa_circ_0000375-TPM2 (I), hsa_circ_0000375-RECK (J), hsa_circ_0000375-AP1S2 (K), hsa_circ_0000375-SRPX (L), hsa_circ_0000375-ABL2 (M), hsa_circ_0000375-ENAH (N), hsa_circ_0000375-SRGAP1 (O) and hsa_circ_0000375-CAP2 (P) pairs in CRC without or with liver metastasis.

CRC with liver metastasis from GSE147597, GSE147602 and GSE147603 were obtained by performing differential expression analysis using the online tool, namely GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/). P-value<0.05 was considered as statistically significant.

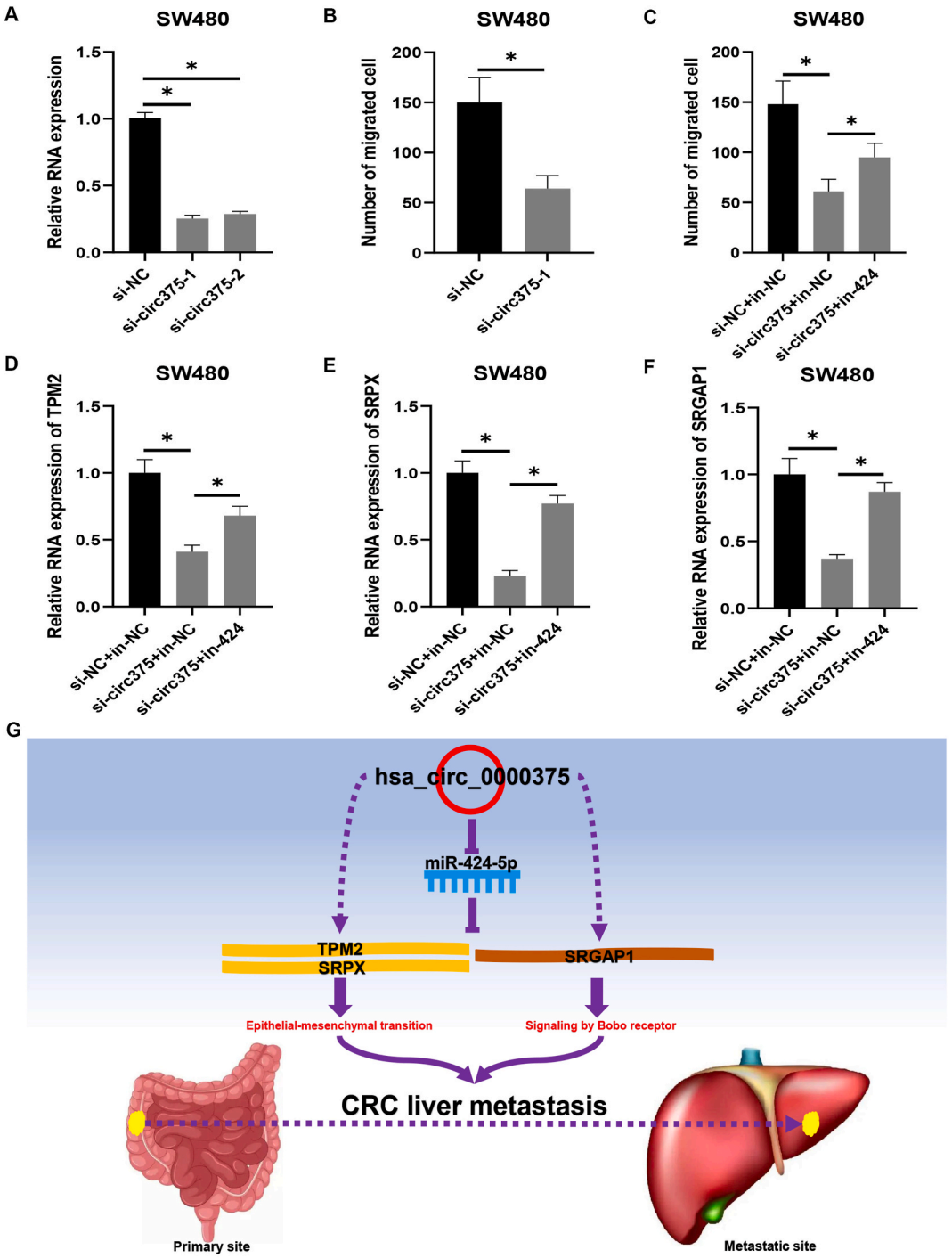

Fig. 12. Construction of a potential circRNA-miRNA-mRNA network contributing to liver metastasis of CRC. (A) The knockdown effect of siRNAs targeting hsa_circ_0000375 in CRC cells. (B) Knockdown of hsa_circ_0000375 reduced the migrated ability of CRC cells. (C) The role of hsa_circ_0000375/miR-424-5p in regulating migration of CRC cells. The role of hsa_circ_0000375/miR-424-5p axis in regulating expression of TPM2 (D), SRPX (E) and SRGAP1 (F) in CRC cells. (G) The mechanistic graph of this work. $^*P < 0.05$.

4.3. *circBase analysis*

circBase (http://www.circbase.org/) [34] is a circRNA-related database, which provides scripts to obtain known and novel circRNAs in sequencing data. circBase was employed to acquire genome location and parental genes of candidate circRNAs.

4.4. Cancer-specific CircRNA database (CSCD) analysis

CSCD (http://gb.whu.edu.cn/CSCD) [35] is a cancer-specific circRNA database, which predicts potential microRNA response element sites, RNA binding protein sites and open reading frames of circRNAs to understand the functional effects of circRNAs. CSCD was introduced to generate the structural patterns of candidate circRNAs.

4.5. miRNA prediction

starBase (http://starbase.sysu.edu.cn/) [36] is a database for decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data, which was utilized to predict possible miRNAs that could potentially bind to candidate circRNAs. The predicted miRNA list could be directly downloaded from the webpage.

4.6. Intersection analysis

Intersection analysis was performed by VENNY 2.1 (http://bioinfogp.cnb.csic.es/tools/venny) tool to obtain the miRNAs that were commonly appeared in "Predicted miRNA" and "DEmiRNA" sets and the genes that were commonly appeared in "Predicted gene" and "DEG" sets.

4.7. Target gene prediction

Target genes of miR-761 or miR-424-5p were predicted using a series of online target gene prediction programs, consisting of PITA, RNA22, miRmap, microT, miRanda, PicTar and TargetScan. To improve the analytic accuracy, only target genes that were appeared in more than 2 programs were selected for subsequent analysis.

4.8. FunRich analysis

FunRich (http://www.funrich.org) [37] is a stand-alone tool used mainly for functional enrichment and interaction network analysis of genes and proteins, which was employed to conduct pathway enrichment analysis for the potential target genes associated with liver metastasis of CRC. The top 10 enriched pathways were downloaded from FunRich tool.

4.9. Correlation analysis

The expression relationship of circRNA-miRNA, miRNA-gene or circRNA-gene pairs in CRC without or with liver metastasis was assessed through performing correlation analysis by usage of GraphPad Prism software. P-value$<$0.05 was considered as statistically significant.

4.9.1. Cell culture and transfection

The human CRC cell line SW480 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences and was maintained in DMEM containing 10 % FBS at 37 °C and 5 % CO_2 atmosphere. The siRNAs targeting hsa_circ_0000375 and its negative control were purchased from Ribobio and were transfected into SW480 cells using Lipofectamine 3000 according to the manufacturer's instructions. 12 h later, culture medium was replaced with fresh medium.

4.9.2. RNA isolation and qRT-PCR analysis

Total RNAs were isolated from SW480 cells using RNAiso plus Reagent, which were reversely transcribed into complementary DNA (cDNA) and qRT-PCR analysis was conducted as we previously described [7]. The expression level of circRNA or gene was finally calculated by the method of 2^{-ddCt}. GAPDH was used as internal control.

4.9.3. Transwell migration assay

Transwell migration assay was employed to detect the migrated ability of SW480. Firstly, 0.05 mL DMEM without FBS and 0.6 mL DMEM with 15 % FBS were added into the upper and lower compartments, respectively. After 30 min later, 0.15 mL serum-free DMEM containing 1 x 10^5 pre-transfected SW480 cells was added into the upper compartment. After incubation for 48 h, the cells on the upper surface were removed, and the cells on the lower surface were successively fixed with 100 % methanol and stained with 0.1 % crystal violet. The cells from five random fields of each insert were counted using a microscope.

4.10. Statistical analysis

Most of the statistical analyses in this study were automatically performed by the online databases or tools as mentioned above. Expression determination between two groups was analyzed by GraphPad Prism software (Version 7). P-value$<$0.05 was considered as statistically significant.

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CRediT authorship contribution statement

Bajin Wei: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Shuyuan Xiao:** Formal analysis, Methodology, Software, Validation, Visualization. **Weiyang Lou:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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